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ORIGINAL ARTICLE

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Comparison of the sulforhodamine B assay and the clonogenic assay for in vitro chemoradiation studies

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Abstract *Purpose*: Since there is a growing interest in preclinical research on interactions between radiation and cytotoxic agents, this study focused on the development of an alternative to the very laborious clonogenic assay (CA). *Methods*: The colorimetric sulforhodamine B (SRB) assay was compared to the clonogenic assay for radiosensitivity testing in two lung cancer cell lines (A549, H292), one colon cancer cell line (HT-29) and one breast cancer cell line (MCF-7). In addition, the combination of the radiosensitizing agent gemcitabine and radiation was investigated with both assays. *Results*: The dose-response curves obtained with the SRB assay and the CA were very similar up to 6 Gy. The radiosensitivity parameters (SF_2, α, β, MID and ID_{50}) obtained from the SRB assay and the CA were not significantly different between H292, A549 and MCF-7 cells. The radiation dose-response curves for A549 and H292 cells pretreated with 4 n*M* gemcitabine for 24 h clearly showed a radiosensitizing effect with both assays. The dose-enhancement factors obtained with the SRB assay and the CA were 1.80 and 1.76, respectively, for A549 cells, and 1.52 and 1.41 for H292 cells. *Conclusions*: The SRB assay was shown to be as useful as the more traditional CA for research on chemotherapy/radiotherapy interactions in cell lines with moderate radiosensitivity. This assay will be used for more extensive in vitro research on radiosensitizing compounds in these cell lines.

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Introduction

As a result of an increasing interest in chemoradiation in the clinic and the development of new agents with radioenhancing potential, there is a growing need for preclinical research on interactions between radiation and chemotherapeutic agents. The clonogenic assay is generally considered the optimal test system for in vitro radiation studies. However, this assay is very time-consuming and laborious, and is open to subjective interpretation due to manual counting. Furthermore, this assay is limited by the need for cells to form colonies and is less reliable for cells with a low colony-forming capacity.

Despite these disadvantages of the clonogenic assay, non-clonogenic assays for radiosensitivity testing have been used in only a few studies [1, 2, 3, 7, 10, 14]. For in vitro studies on the interactions between radiotherapy and chemotherapy one could argue that colorimetric assays would be preferable because these assays are more rapid, can be semiautomated and have a greater capacity to test different treatment conditions in one experiment. Indeed for chemosensitivity testing, the colorimetric assays, such as the tetrazolium (MTT) and the sulforhodamine B (SRB) assays, have replaced the clonogenic assay. However, for radiosensitivity testing the clonogenic assay is still the gold standard.

The colorimetric assays are thought to be inadequate to measure radiation sensitivity, due to the short duration of the assays. After radiation treatment, cells destined to die can still undergo one or more cell divisions. Therefore, it takes a considerable period of time before these irradiated cells express their radiation-induced damage.

Negative aspects of the MTT assay, which is based on the ability of viable cells to reduce 3-(4,5-dimethylthiazol-

2-yl)-2,5,diphenyltetrazolium bromide into blue-purple formazan crystals, are the need for refeeding during long incubation periods and an overestimation of the survival rate when control nonirradiated cells reach confluence. On the other hand, short durations of the experiment might result in an overestimation of cell survival due to delayed expression of radiation damage. Furthermore, optimal seeding densities and assay duration require optimization of the conditions for each cell line [3, 14].

For chemosensitivity testing, the SRB assay, which is based on the staining of cellular proteins, has many advantages over the MTT assay. The SRB assay shows a wide linear range with cell number, while the MTT test is not linear at high cell densities [9]. In contrast to the MTT assay, SRB staining is not dependent on mitochondrial activity, and this results in a low variation between cell lines and therefore there is less need for cell line-specific optimization. Although both assays are comparable in terms of outcome when used for chemosensitivity testing [6, 8, 9, 15], the SRB assay is preferred for its lower variation between cell lines, higher sensitivity and larger linear range [8, 9, 15]. Moreover, the SRB assay has some practical advantages over the MTT assay, i.e. the staining is stable for a long period of time and the assay can be interrupted at several steps during the staining protocol [6, 8, 15].

The use of the SRB assay for the measurement of cell survival after irradiation has been very limited. In only two studies the SRB, MTT and clonogenic assays have been compared for radiosensitivity testing [1, 7]. Griffon et al. reported a moderate to good correlation between the clonogenic assay and the SRB assay and found no significant differences between the two assays for radiation doses up to 6 Gy [7]. However, Banasiak et al. have claimed that the colorimetric assays are relatively insensitive at lower radiation doses [1].

Because of the growing need for less laborious and more rapid methods for in vitro research on combinations of chemotherapy and radiotherapy, we investigated whether the SRB assay would be a suitable method. Since our in vitro research on radiosensitizers is mainly focused on cell lines with moderate to low radiosensitivity, we determined the radiosensitivity of four moderately radiosensitive cell lines by the SRB assay and, for comparison, by the clonogenic assay. In addition, the combination of radiation and the radiosensitizing agent gemcitabine was investigated using both assays.

Materials and methods

Cell lines

The cell lines used in this study were H292 and A549, two lung cancer cell lines, HT-29, a colon cancer cell line, and MCF-7, a breast cancer cell line. H292 and A549 cells were cultured in RPMI-1640 medium, supplemented with glutamine, sodium pyruvate and 10% fetal calf serum (Invitrogen, Merelbeke, Belgium). HT-29 and MCF-7 were cultured in DMEM supplemented with glutamine and 10% fetal calf serum. Cultures were incubated at 37°C under an atmosphere comprising 5% CO_2/95% air.

Clonogenic assay

The standard clonogenic assay was performed as previously described [11, 12]. Briefly, cells were harvested from exponential phase cultures by trypsinization, counted and plated in six-well plates. Seeding densities varied from 250 to 1800 cells per well for H292, A549 and HT-29 cells depending on the planned radiation dose. The plating efficiency was consistent and varied between the cell lines from 50% to 70%. For MCF-7 cells, which had a plating efficiency of only 15%, the densities varied from 1000 to 6500 cells per well. Two seeding densities per radiation dose were used in triplicate (total six wells per dose).

Cells were irradiated at room temperature over the dose range 0–8 Gy, using a ^{60}Co source (St. Augustinus Hospital, Antwerp). Thereafter, the plates were incubated without refeeding for a period sufficient to form colonies of at least 50 cells (i.e. six cell doubling times). For all four cell lines, 7 days of incubation after radiation was needed to form colonies. After incubation, the medium was aspirated and cells were washed with 0.9% NaCl. Clones were stained with crystal violet (2 g/l in 6% glutaraldehyde) and counted under a microscope by two independent investigators. Only clones containing more than 50 cells were considered to be colonies. The mean of the two counts was used for further calculations.

SRB assay

Cells were harvested from exponential phase cultures by trypsinization, counted and plated in 48-well plates. Optimal seeding densities for each cell line were determined to ensure exponential growth during a 7-day assay. Seeding densities were 400, 150, 70 and 600 cells per well for H292, A549, HT-29 and MCF-7 cells, respectively, independent of the planned radiation dose. Plates were irradiated at room temperature over the dose range 0–8 Gy using a ^{60}Co source. Thereafter, cells were incubated for 7 days before determination of the survival by the SRB assay.

The SRB assay was performed according to the method of Skehan et al. and Papazisis et al, with minor modifications [13, 16]. The culture medium was aspirated prior to fixation of the cells by the addition of 200 µl 10% cold trichloroacetic acid. After a 1-h incubation at 4°C, cells were washed five times with deionized water. The cells were then stained with 200 µl 0.1% SRB (ICN, Asse, Belgium) dissolved in 1% acetic acid for at least 15 min and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were left to dry at room temperature and bound protein stain was solubilized with 200 µl 10 m*M* unbuffered Tris base (tris(hydroxymethyl)aminomethane) and transferred to 96-well plates for reading the optical density (OD) at 540 nm (Biorad 550 microplate reader, Nazareth, Belgium).

Combination of radiotherapy and gemcitabine

In A549 and H292 cells, the radiosensitizing effect of gemcitabine (2′,2′-difluorodeoxycytidine) was investigated by the clonogenic and the SRB assays. Cells were plated in 6- or 48-well plates, as described above. Three or six wells of the 6- or 48-well plates, respectively, were treated with 4 n*M* gemcitabine in phosphate-buffered saline (PBS). PBS was added to the control cells. After a 24-h incubation, the plates were irradiated and immediately washed with drug-free medium. After 7 days, cell survival was determined by the clonogenic assay or the SRB assay.

Statistical methods

For the clonogenic assay, survival rates were calculated as: (mean plating efficiency of treated cells/mean plating efficiency of control cells)×100%, where the plating efficiency is the number of colonies divided by the number of inoculated cells. For the SRB assay, the survival curves were calculated as: (mean OD of treated cells/mean

OD of control cells)×100%. The radiation survival curves were fitted according to the linear-quadratic model using WinNonlin (Pharsight, Mountain View, Calif.): survival = $\exp(-\alpha D - \beta D^2)$.

The following parameters were calculated: the linear component α, which represents single-hit killing kinetics and dominates at low radiation doses; the quadratic component β, which causes the curve to bend at higher doses; ID_{50}, the radiation dose causing 50% growth inhibition; SF_2, the surviving fraction at 2 Gy; and the mean inactivation dose (MID), which was calculated by numerical integration of the linear-quadratic curve [5]. For the calculation of the survival curves after treatment with radiotherapy in combination with gemcitabine, the survival rates were corrected for the cytotoxic effect of gemcitabine itself. The radiosensitizing effect of gemcitabine was calculated in terms of the dose-enhancement factor (DEF): ID_{50}(control)/ID_{50}(gemcitabine-treated).

True radiosensitization can be defined as a synergistic interaction between gemcitabine and radiation. For the determination of synergism, the combination index (CI) was calculated by the Chou-Talalay equation [4, 12], using CalcuSyn (Biosoft, Cambridge, UK). The CI values obtained from the classic (mutually exclusive) isobologram analysis are given. CI values between 0.9 and 1.1 indicate only additivity. Moderate synergism is indicated by CI values between 0.7 and 0.9, and synergism by CI values below 0.7.

The results are expressed as mean ± standard deviation. All experiments were performed at least three times. A two-sample *t*-test was used to determine whether the parameters of the dose-response curves of each cell line obtained from the two assays were significantly different.

Results

Dose-response curve characteristics

Radiation dose-response curves for the four cell lines, as measured by the clonogenic and SRB assays are illustrated in Fig. 1. Each curve represents the mean of three experiments. The curves obtained from the SRB and clonogenic assays were very similar up to 6 Gy. After treatment with 8 Gy, a lower survival rate was observed with the clonogenic assay in comparison with the SRB assay. The differences were most pronounced in HT-29 and MCF-7 cells.

Radiosensitivity parameters obtained by the SRB and clonogenic assays

The values of dose-response curve parameters (α, β, ID_{50}, SF_2 and MID) calculated from the two assays are listed in Table 1. The radiosensitivity parameters obtained from the SRB and clonogenic assays were not significantly different between the H292, A549 and MCF-7 cells ($P = 0.23$–0.94, two-sample *t*-test for independent samples). Only for the HT-29 cells was a significant difference observed for the ID_{50} and MID values.

Combination of radiotherapy and gemcitabine

Radiation dose-response curves for A549 and H292 cells pretreated with or without 4 n*M* gemcitabine, measured by the clonogenic and SRB assays, are shown in Fig. 2. Since the survival curve of radiotherapy plus gemcitabine was corrected for the cytotoxic effect of gemcitabine itself (survival 94% for A549, 98% for H292), comparison of the curves clearly shows that gemcitabine enhanced the radiosensitivity of the cells in both assays. For A549, the enhancement effect was moderately synergistic (mean CI 0.80 in the SRB assay and 0.67 in the clonogenic assay). In H292 cells, 4 n*M* gemcitabine resulted in only an additive effect, but the same trends were observed as for A549 (mean CI 0.89 in the SRB assay and 0.93 in the clonogenic assay). Radiosensitivity parameters and DEFs are shown in Table 2. The ID_{50}, SF_2 and MID of A549 cells were significantly lower after treatment with gemcitabine ($P < 0.02$ for both assays). This was also indicated by a significant increase in the α value ($P < 0.05$ for both assays). For H292, only the ID_{50} was significantly different after treatment with 4 n*M* gemcitabine ($P = 0.044$) when determined by the SRB

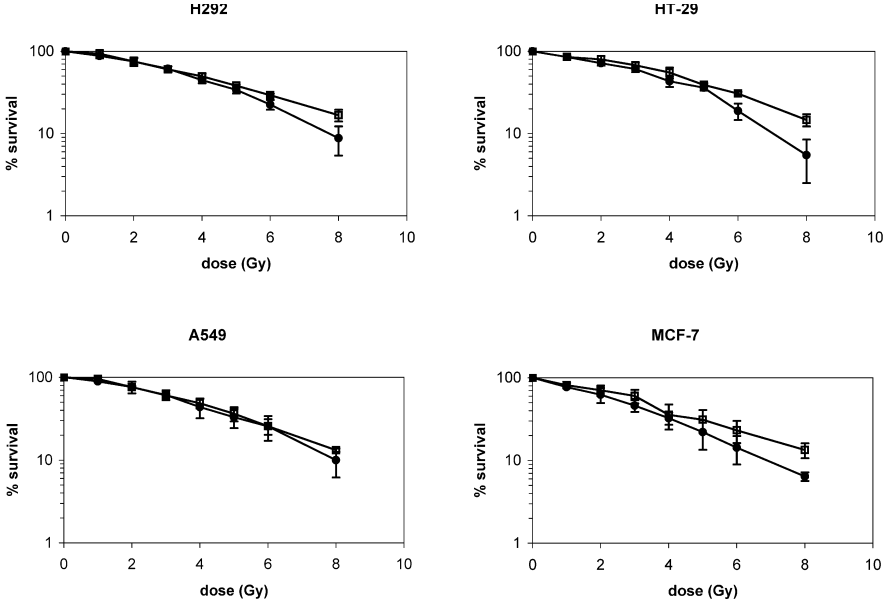

Fig. 1 Dose response curves of H292, A549, HT-29 and MCF-7 cells treated with radiotherapy, obtained with the SRB assay (□) and the clonogenic assay (●). Experiments were performed at least three times, and the results are expressed as mean ± standard deviation

Table 1 Radiosensitivity parameters obtained by the clonogenic and SRB assays in fourdifferent cell lines treated with radiotherapy over the dose range 0–8 Gy.The results are expressed as mean ± standard deviation of at least three experiments. For abbreviations, see text

Radiosensitivity parameter	Assay	Cell line			
		H292	A549	HT-29	MCF-7
α (Gy^{-1})	Clonogenic	0.091 ± 0.076	0.087 ± 0.048	0.103 ± 0.028	0.215 ± 0.080
	SRB	0.108 ± 0.071	0.078 ± 0.040	0.093 ± 0.060	0.160 ± 0.074
β (Gy^{-2})	Clonogenic	0.027 ± 0.013	0.027 ± 0.012	0.026 ± 0.006	0.018 ± 0.004
	SRB	0.017 ± 0.012	0.026 ± 0.011	0.017 ± 0.010	0.015 ± 0.005
ID_{50} (Gy)	Clonogenic	3.68 ± 0.63	3.74 ± 0.42	3.56 ± 0.24	2.70 ± 0.55
	SRB	3.96 ± 0.43	3.93 ± 0.54	4.19 ± 0.31*	3.37 ± 0.80
SF_2 (%)	Clonogenic	75.1 ± 8.6	75.5 ± 5.0	73.4 ± 3.1	61.0 ± 8.4
	SRB	75.7 ± 6.9	77.2 ± 5.1	77.8 ± 6.3	68.8 ± 9.4
MID (Gy)	Clonogenic	4.14 ± 0.55	4.18 ± 0.50	4.01 ± 0.23	3.29 ± 0.51
	SRB	4.64 ± 0.08	4.38 ± 0.58	4.79 ± 0.09*	4.01 ± 0.71

*$P < 0.05$ compared to CA

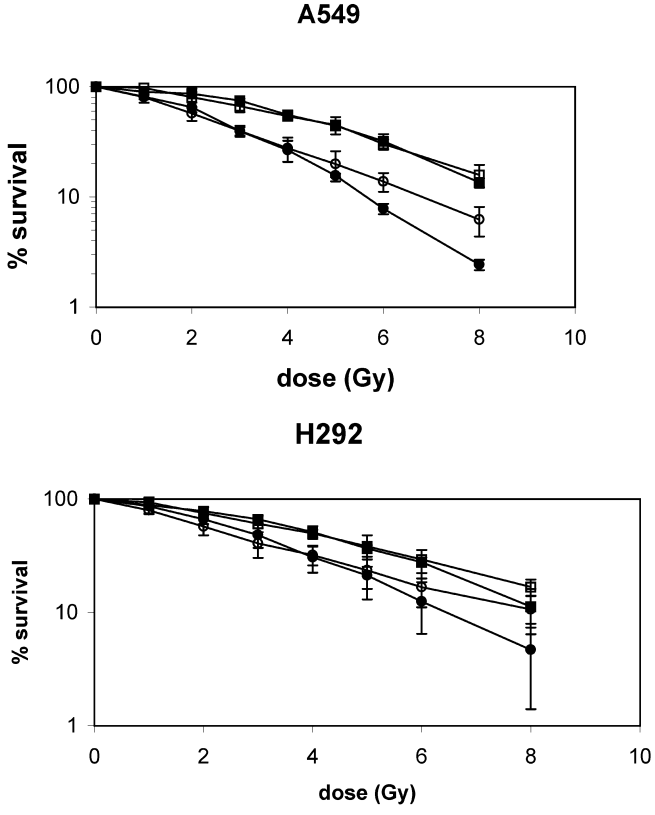

Fig. 2 Dose response curves of A549 and H292 cells treated with (○, ●) or without (□, ■) 4 n*M* gemcitabine 24 h prior to radiation, obtained with the SRB test (○, □) and the clonogenic assay (●, ■). Experiments were performed at least three times, and the results are expressed as mean ± standard deviation

assay. The other radiosensitizing parameters were not significantly different ($P > 0.05$ for both assays). The radiosensitizing effect was calculated in terms of the DEF, which was 1.80 for A549 cells in the SRB assay. This was comparable to the DEF derived from the clonogenic assay (1.76). Also for H292, the DEFs derived from both assays were comparable: 1.52 and 1.41 for the SRB assay and clonogenic assay, respectively. The radiosensitizing effect was clearly shown by both assays by a decrease in ID_{50}, SF_2 and MID, mainly caused by an increase in the α value.

Only for high radiation doses in combination with gemcitabine were lower survival rates observed with the clonogenic assay in comparison to the SRB assay.

Discussion

Our study showed that, in the four cell lines tested, the SRB assay was a suitable test system for in vitro radiosensitivity testing and that, in the relevant dose range, it was clearly comparable in terms of outcome to the clonogenic assay, which so far has been the gold standard [11, 12]. Moreover, our study indicated that this test system has major advantages over the clonogenic assay for interaction studies of radiotherapy and chemotherapy in cell lines with moderate radiosensitivity. Cell survival in in vitro experiments with radiotherapy is traditionally determined with the clonogenic assay. This assay is very laborious and does not allow survival of cells with no or low clonogenic capacity to be measured. Colorimetric assays, which can overcome these limitations, have generally been thought to be unsuitable for the measurement of radiosensitivity.

Discrepancies observed by others between the clonogenic assay and the MTT assay have been explained by the difference in end-points of the two assays. Cells which have lost their reproductive potential, but which are still viable will be recorded by the MTT assay, but not by the clonogenic assay [1]. Furthermore, dead cells may still retain residual dehydrogenase activity, on which the MTT assay is based [3] and mitochondrial activity can be inhibited at high cell densities [14]. These latter explanations for the discrepancy between the MTT assay and the clonogenic assay are not applicable to the SRB assay. Furthermore, the SRB assay has several practical advantages over the MTT assay.

The duration of the assay turned out to be an important factor in the suitability of both the MTT assay and the SRB assay for radiosensitivity testing [7]. Since radiation-induced damage is observed only after one or

Table 2 Radiosensitivity parameters and dose-enhancement factors obtained by the SRB and the clonogenic assays in A549 and H292 cells treated with or without 4 n*M* gemcitabine 24 h prior to radiation over the dose range 0–8 Gy. The results are expressed as mean ± standard deviation of at least three experiments. Forabbreviations, see text

Radiosensitivity parameter	Concentration ofgemcitabine (n*M*)	A549		H292	
		SRB	CA	SRB	CA
α (Gy^{-1})	0	0.059 ± 0.060	0.030 ± 0.020	0.108 ± 0.071	0.073 ± 0.032
	4	0.246 ± 0.086	0.143 ± 0.019	0.252 ± 0.087	0.129 ± 0.076
β (Gy^{-2})	0	0.023 ± 0.006	0.027 ± 0.003	0.017 ± 0.012	0.025 ± 0.008
	4	0.017 ± 0.011	0.048 ± 0.007*	0.005 ± 0.008	0.036 ± 0.011
ID_{50} (Gy)	0	4.37 ± 0.58	4.55 ± 0.23	3.96 ± 0.43	4.07 ± 0.55
	4	2.45 ± 0.44	2.60 ± 0.16	2.69 ± 0.59	3.29 ± 0.19
SF_2 (%)	0	81.4 ± 7.8	84.6 ± 2.7	75.7 ± 6.9	78.3 ± 5.1
	4	57.5 ± 7.4	62.0 ± 2.8	59.7 ± 8.2	66.3 ± 9.7
MID (Gy)	0	4.85 ± 0.46	4.94 ± 0.23	4.64 ± 0.08	4.51 ± 0.62
	4	3.05 ± 0.37	2.90 ± 0.17	3.51 ± 0.59	3.26 ± 0.55
DEF (ID_{50})		1.80 ± 0.12	1.76 ± 0.12	1.52 ± 0.23	1.41 ± 0.10

*$P < 0.05$ compared to SRB

more cell divisions, the assay duration should be adapted to the doubling time of the cells. Carmichael et al. have shown that the results of the MTT assay are comparable to those of the clonogenic assay when the duration of the MTT assay is extended to at least six control cell doubling times [3].

Thus, it seems essential that sufficient time is allowed for the cells to die after they are damaged by radiation. However, to avoid an overestimation of survival, control cells have to remain in exponential growth up to the end of the assay. The study by Banasiak et al. suggests that the MTT and SRB assays are less sensitive at lower radiation doses, as indicated by a large shoulder in the radiation dose-survival curve [1]. Since the assay duration was 14 days, these observations could be explained by the fact that confluence was reached in their assays, resulting in an overestimation of survival, especially at lower radiation doses.

In our study, the SRB assay was compared to the clonogenic assay for studying radiosensitivity of four cell lines, two lung cancer cell lines, a colon cell line and a breast cancer cell line. To allow sufficient time for the delayed radiation-induced cell death, the duration of the assay should be equivalent to at least six cell doubling times. The duration of he SRB assay was adapted to the duration of the clonogenic assay (at least six doubling times, which is the time needed to form colonies of at least 50 cells). The cell lines used in our study had comparable doubling times and for all four cell lines 7 days was sufficient to form colonies. To prevent confluence during the 7 days of the assay, the number of cells plated at the start of the SRB assay was limited. To overcome a decrease in reliability due to very low cell concentrations, the cells were seeded in 48-well plates instead of 96-well plates. This created a broader margin for longer incubation times or higher cell concentrations without confluence or the need for refeeding.

The radiation dose-survival curves obtained with this SRB assay were comparable to those obtained with the clonogenic assay for all four cell lines. Only at very high radiation doses of 8 Gy were lower survival rates shown in the clonogenic assay in comparison to the SRB assay. This might be an artifact of the clonogenic assay caused by the relatively high cell concentration used at high radiation doses. High cell concentrations might increase the incidence of cell clusters, resulting in a lower number of colonies. This hypothesis is supported by the fact that differences were most pronounced in HT-29 and MCF-7 cells, which both have a tendency to cluster. Another explanation might be that after higher doses of radiation, SRB staining may start to approach background levels, which might result in an upward bending of the curve. While the clonogenic assay can be used to address survival levels at several decades of depopulation, this might be a problem for the SRB assay. Since the results of the SRB assay were highly comparable to those of the clonogenic assay at low, clinically more relevant, doses (0–6 Gy), the SRB assay would be suitable for in vitro radiosensitivity testing in moderately radiosensitive cell lines. For experiments with higher radiation doses, accumulated doses or more radiosensitive cell lines, again a comparison with the clonogenic assay should be made before application of the SRB assay. The SRB assay is less useful for primary samples, since it is important that the duration of the assay is adapted to the doubling time of the cells and the number of cells plated should be optimized to prevent confluency during the experiment.

It should be emphasized that the SRB assay offers several practical advantages over the clonogenic assay, especially for research on combinations of radiotherapy and chemotherapy. Since the SRB assay is semiautomated and offers a greater opportunity to test different treatment conditions in one experiment, it is well suited to in vitro research on chemotherapy/radiotherapy interactions. We investigated the radioenhancement effect of gemcitabine by both the SRB assay and the clonogenic assay. A clear radiosensitizing effect of gemcitabine was observed, which was comparable between the two test systems. Slight differences were observed at high radiation doses in combination with gemcitabine. This

might again be explained by cell clusters at high cell concentrations or the fact that SRB staining may start to approach background levels. Since radioenhancing agents such as gemcitabine clearly have a radiosensitizing effect at low radiation doses, by increasing the α value of the linear quadratic model, the SRB assay is well suited to the investigation of radiosensitization. Therefore, the SRB assay is now being used in our laboratory for further research of the combination gemcitabine/radiotherapy.

In conclusion, when sufficient time is allowed for delayed radiation-induced cell death and exponential growth is guaranteed during the assay, the results of the SRB assay are highly comparable to those obtained with the "gold standard" clonogenic assay in moderately radiosensitive cell lines. Only at high, clinically less-relevant, radiation doses were some minor differences shown. The application of the SRB assay for radiosensitivity testing in more radiosensitive cell lines still has to be investigated. The SRB assay was also shown to be applicable for research on chemotherapy/radiotherapy interactions and offers the opportunity for more extensive in vitro research on radiosensitizing compounds.

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